In Vitro Tissue Culture of Pear: Advances in Techniques for Micropropagation and Germplasm Preservation

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Abstract

Micropropagation techniques for over 20 pear (*Pyrus*) cultivars belonging to seven species have been reported. While most published methods use Murashige and Skoog (MS) basal nutrient medium, or slight modifications thereof, Lepoivre (LP) and Driver-Kuniyuki Walnut (DKW) media, which differ from MS in nitrogen concentration or source, and calcium concentration, have improved shoot proliferation rates. Solid media gelled with agar, sometimes in combination with gellan gum, have traditionally been used, but two-phase liquid overlay or intermittent liquid immersion techniques have greatly increased shoot proliferation. In vitro culture methods, including meristem cryopreservation, are important facets of medium-term and long-term germplasm preservation programs. Medium-term (1 to 4 years) storage techniques involve temperatures of 1 °C to 4 °C, usually in reduced light or darkness, in a nutrient medium with no growth regulators. Long-term preservation of meristems can be accomplished by one of three major methods of cyropreservation: slow freezing, vitrification, and encapsulation-dehydration. Slow freezing, combined with pretreatment by either cold acclimation or abscisic acid has proven to be particularly effective for *Pyrus* germplasm, including cold-tender species.

INTRODUCTION

The ability to establish shoot tip cultures, proliferate shoots, induce rooting, and acclimatize the resulting plantlets are all elements of in vitro micropropagation. The methods offer an alternative to the propagation of rootstocks by stooling, layerage, or induced rooting of softwood, semi-hardwood, or hardwood cuttings (Hartmann et al., 1997; Howard, 1987). These same methods could also be used to produce self-rooted scion cultivars. General and genotype-specific protocols that have been empirically developed for pear have been the subject of previous reviews (Chevreau et al., 1992; Chevreau and Skirvin, 1992; Hutchinson and Zimmerman, 1987; Singha, 1986). Meristem culture has been used to produce pathogen-free plants following thermotherapy, and for cryopreservation of pear germplasm (Reed, 1990). In vitro micropropagation methods are also general prerequisites to exploiting somaclonal variation and induced mutations, and for the development of transgenic plants.

MICROPROPAGATION

Species and Genotypes

Micropropagation protocols have been published, beginning in the late 1970's, for over 20 cultivars of pear, including the major *Pyrus communis* cultivars, but also several Japanese cultivars of *P. pyrifolia* (Burm. f.) Nakai (Bhojwani et al., 1984), plus genotypes of *P. amygdaliformis* Villars (Dolcet-Sanjuan et al., 1990), *P. calleryana* Decne. (Berardi et al., 1992), *P.* x *bretschneideri* Rehder (Chevreau et al., 1989), *P. pyraster* L. (Damiano et al. 1996), *P. syriaca* Boiss. (Shibli et al., 1997), and *P. betulifolia* Bunge (Nicolodi and Pieber, 1989; Dolcet-Sanjuan et al., 1990), as well as for a quince (*Cydonia oblonga* L.) rootstock (Dolcet-Sanjuan et al., 1990). Empirical studies to determine optimum cultivar-specific protocols have been conducted for some, but not all of these genotypes: 'Bartlett' (Lane,

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1979; Shen and Mullins, 1984), 'Old Home' x 'Farmingdale' 51 rootstock (Cheng, 1979), 'Beurré Bosc' (Shen and Mullins, 1984; Bell et al., 1999), 'Packham's Triumph' (Shen and Mullins, 1984), 'Passe Crassane' (Al Maarri et al., 1986; Leblay et al., 1991), 'Conference' (Predieri et al., 1989; Leblay et al., 1991; De Paoli, 1989), 'Louise Bonne' (Chevreau et al., 1989), ' Doyenne du Comice' (De Paoli, 1989; Leblay et al., 1991), 'Crystal' (Chevreau et al., 1989), and 'Hosui' and four other *Pyrus pyrifolia* cultivars (Bhojwani et al., 1984; Hirabayashi et al., 1987).

Shoot Proliferation Protocols

In vitro establishment methods are fairly standard, with little variation. More variation exists in shoot proliferation media. The major factors are (1) macro- and micronutrient composition of the medium, and (2) phytohormones. Of eleven major media reported, most used Murashige and Skoog (MS) (Murashige and Skoog, 1962) plant tissue culture medium, five without modification, and 2 with differences in the nitrogen concentration and type. Only a few of the studies made comparisons among many basal media, so general conclusions are difficult to make. Nedelcheva (1986) and Al Maarri et al. (1986) found that proliferation of 'Bartlett' and 'Passe Crassane' was greatest on Lepoivre (LP) medium (Quoirin and Lepoivre, 1977). In another study, DKW (Driver and Kuniyuki, 1984) has resulted in greater shoot proliferation than MS, LP, and Woody Plant Medium (WPM) (Lloyd and McCown, 1981) for 'Beurré Bosc', and is at least as effective for 'Bartlett' as MS-based media (Bell et al., unpublished data). The major differences in macronutrients among these four basal media are in ammonium and nitrate ion concentrations and total ion concentration. Full-strength MS is highest in ammonium and nitrate, followed by DKW, while LP is a low ammonium medium. Both LP and DKW have calcium nitrate as a major nitrogen source, and DKW is highest in calcium.

Benzyladenine (BA) is the cytokinin of choice for inducing axillary bud growth. Zeatin and 2-isopentenyladenine (2-iP) have been rarely used, for example, as supplements to BA (Shen and Mullins, 1984). Use of 2-iP tends to result in larger leaves, but in the absence of BA, results in decreased axillary budbreak (Bell, 1995; Moretti et al,, 1992). Concentrations of BA range from 2.2 M to 10 M.

Auxins have been used in most protocols. Eight of the media used indole butyric acid (IBA) at 0.5 M, three media used naphthelene acetic acid (NAA), and only three reported no use of auxin (Lane, 1979; Shen and Mullins, 1984; and Singha, 1980). Two media contained the gibberellin, GA_3 at 0.3 or 0.6 M. However, Rodriguez et al. (1991) suggest that GA_3 inhibited shoot proliferation in 'Jules Guyot' and 'Butirra Precoce Morettini', and our own data indicates that GA_3 may inhibit shoot proliferation of 'Bartlett', but not 'Beurré Bosc'. Rodriguez et al. (1991) also suggested that subsequent rooting was inhibited.

Agar, usually at 6 g L^{-1}, is used predominantly as a medium gelling agent to provide support to cultured tissues. A mixture of agar and gellan gum (Phytagel™ or Gelrite™) has been used at the National Clonal Germplasm Repository (NCGR) for germplasm storage, primarily as a cost saving measure. De Paoli (1989) used a combination of agar and pectin. Gellan gum alone tends to result in hyperhydricity of cultured tissue. Zimmerman et al. (1995) reported that the combination of corn starch and gellan gum resulted in hyperhydricity and poor growth of 'Seckel' and 'Beurre d'Anjou'. Addition of a hydric control agent (HCA), was successful in eliminating hyperhydricity. The use of a gellan gum-starch mixture as a low cost alternative to agar was, therefore, recommended, if also combined with an appropriate HCA.

Reported shoot multiplication rates have usually not exceeded 4-6 shoots per original explant. However, Bommineni et al. (2000) developed a rapid shoot multiplication technique involving preconditioning in high concentrations of cytokinins to produce shoots with very short internodes with enlarged meristems, followed by thin-sectioning to divide the meristems. Culturing the thin shoot slices on a shoot induction medium produced multiple meristems which developed into shoots.

Another promising technique is the use of a double-phase culture system, first reported by Viseur (1987), in which an agar base is overlaid with liquid medium. More

recently, Rodriguez et al. (1991) demonstrated the effectiveness of this system with 'Abbé Fétel', 'Jules Guyot', and ' Butirra Precoce Morettini', achieving multiplication rates as high as 15 shoots per explant. The double-phase system has become popular with commercial propagators in the United States. An automated temporary (30 to 60 minutes per day) liquid immersion system has resulted in excellent shoot proliferation rates for *Pyrus communis* var. *pyraster* (Damiano et al. (1996) and 'Conference', 'Precoce di Fiorano', and 'Harvest Queen' (Damiano et al., 2000), with neither the hyperhydricity often associated with liquid culture, nor the apical necrosis sometimes observed on solid media.

Rooting Protocols

Rooting of in vitro produced shoots has changed little over the last 20 years. The two basic methods involve (1) a low concentration of auxin incorporated in semi-solid medium for a minimum of 1 week, and (2) a quick (10 s to 1 min) dip in a high concentration of auxin. In the former method, NAA or IBA at 0.1- 10 M, or IAA at 10 - 11 M have been used, while quick dip methods have used IBA at 10mM or 2000 mg L^{-1} concentrations. The basal medium has typically been MS or half-strength MS. Light exclusion is typically not used, but may increase rooting. Yeo and Reed (1995) investigated several methods and found rooting frequencies of 28 to 100% for 49 genotypes, depending on the treatment and genotype. Damiano et al. (2000) reported inconsistent results with temporary immersion in rooting media containing the gibberellin inhibitors phloroglucinol, paclobutrazol, or flurprimidol. Damiano and Monticelli (1998) demonstrated that in vitro rooting of *P. pyraster* could be improved by treatment with *Agrobacterium rhizogenes.*

IN VITRO PRESERVATION OF PEAR GERMPLASM

Primary collections of plant germplasm are often in field plantings that are vulnerable to disease, insect, and environmental stresses. Alternative germplasm storage technologies provide a secondary storage method for clonal field collections, storage for experimental material, allow for staging of commercial tissue culture crops, and provide a reserve of germplasm for plant distribution. Cryopreservation in liquid nitrogen (LN) allows storage of a base collection (long-term backup) of clonal materials. Cryopreservation methods are now well developed and make these long-term collections of clonal germplasm feasible. Both in vitro and cryopreserved collections provide insurance against the loss of valuable genetic resources and may provide alternative distribution methods.

In Vitro Storage

Medium-term storage of clonal plants involves strategies to slow growth through temperature reduction, environmental manipulation, or chemical additions in the culture medium (Wanas et al., 1986). Several laboratories have developed strategies for slow-growth storage of pears. A standard storage treatment for *Pyrus communis* and many other species is 4 °C with a 16-h photoperiod for 12 to 18 months. In comparison, the Japanese pears, *P. pyrifolia,* survive best when stored at 1 °C in the dark for 12 months (Moriguchi et al., 1990; Moriguchi, 1995). Pears stored at NCGR-Corvallis in 1984 were successfully stored in 20 x 100 mm tubes at 4 °C in darkness, but tubes were replaced with gas-permeable tissue-culture bags in 1989. *Pyrus* accessions (n = 169) stored in these tissue-culture bags in the dark at 4 °C had a mean storage life of 2.7 years (range from eight months to 4.6 yr) (Reed and Chang, 1997). Three cold-storage treatments (1^{o}C upright plants, 4^{o}C 3/4 submerged, 1 °C upright) revealed differences among the 46 genotypes for storage duration, but few differences were noted among the treatments for a single genotype (Reed et. al., 1998b).

Cryopreservation

New techniques and improvements in cryopreservation research have greatly increased the number of cryopreserved species. Suspension or callus cultures, dormant buds, in vitro-grown apical meristems, isolated embryonic axes, seeds, somatic embryos, and pollen are now stored in LN. Dormant bud cryopreservation started with Sakai's early experiments on fruit trees (summarized in Sakai, 1985). More recent work with dormant,

cold-hardy *Pyrus* shoots has been very successful (Moriguchi et al., 1985; Oka et al., 1991; Mi and Sanada, 1992; 1994).

The three major meristem cryopreservation techniques, slow freezing, vitrification, and encapsulation-dehydration, provide options for most types of plant materials (Benson, 1999). In vitro-grown pear shoot meristems were first successfully cryopreserved in 1990 (Dereuddre et al., 1990a; b; Reed, 1990). A slow freezing method for in vitro-grown pear meristems 55% to 95% regrowth of four *Pyrus* species including a subtropical species, *P. koehnei* (Reed, 1990). Encapsulation-dehydration was applied to pear by Dereuddre et al., (1990a; b). A 0.75 M sucrose preculture and 4-hr dehydration (20% residual water) produced 80% recovery (Scottez et al., 1992). A modified encapsulation-dehydration method developed by Niino and Sakai (1992) produced 70% recovery, and their vitrification method 40% to 72.5% regrowth (Niino et al., 1992; Suzuki et al., 1997). A comparison of slow freezing and vitrification methods using 28 *Pyrus* genotypes found that 61% had better than 50% regrowth following slow freezing (0.1 °C/min), while only 43% responded this well to the vitrification technique (Luo et al., 1995; Reed et al., 1998a).

Recent studies show that pretreatment of stock plants with cold acclimation (CA) or abscisic acid (ABA) is very important for cryopreservation of many pear genotypes. Alternating-temperature (22 °C for 8-12 h/-1°C for 12-16 h) CA for 2 to 5 weeks significantly increases regrowth, and recovery remains high for shoots with up to 15 weeks of CA. Constant temperature acclimation is less effective (Chang and Reed, 2000). Abscisic acid in the preculture medium shortens the acclimation period from 10 days to 2 weeks for *P. cordata*, a particularly difficult taxon to cryopreserve (Chang and Reed, 2001).

CONCLUSIONS

Fortunately, in vitro culture and cryopreservation have progressed to the point where they can be used routinely in many laboratories. Important advances have been made in proliferation, particulary with the development of liquid immersion techniques, and in genotype-specific rooting protocols. In vitro-stored plantlets are used as primary or duplicate collections in several facilities. Long-term (base) storage of important collections through cryopreservation of meristems is now a reality as well (Reed et al., 1998b; Reed, 1999). Cryopreserved meristems provide an important, previously missing, form of long term germplasm storage for vegetatively propagated plants. The greatest cost of cryopreservation is in the initial storage of an accession, but very little input is needed for many years after storage. Initial storage costs are often similar to the cost of maintaining accessions in the field for one year. Costs for maintenance in LN are minimal. Although plants can be distributed as cryopreserved samples, they are best kept as insurance in case of loss of actively growing accessions.

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